U.S./CANADA TRANSBOUNDARY RIVER ENHANCEMENT PROJECT: CONTRACT REPORT FOR THE PERIOD FEBRUARY 1991 TO JULY 1993

By

Peter Hagen

Regional Information Report[1] No. 1J93-18

Alaska Department of Fish and Game
Commercial Fisheries Management and Development Division
Juneau, Alaska

July 1993

---

[1] The Regional Information Report Series was established in 1987 to provide an information access system for all unpublished divisional reports. These reports frequently serve diverse ad hoc informational purposes or archive basic uninterpreted data. To accommodate timely reporting of recently collected information, reports in this series undergo only limited internal review and may contain preliminary data; this information may be subsequently finalized and published in the formal literature. Consequently, these reports should not be cited without prior approval of the author or the Commercial Fisheries Management and Development Division.

AUTHOR

Peter T. Hagen is the Transboundary River Enhancement Project Leader, directing the Otolith Laboratory for the Department of Fish and Game, Commercial Fisheries Management & Development Division, Southeast Region, P.O. Box 240020, Douglas, Alaska 99824-0020.

ACKNOWLEDGEMENTS

Regional staff from the Division of Commercial Fisheries Management & Development who assisted in this project include; Kristen Munk, Ben Van Alen, Andy McGregor, Kathleen Jenson, Carol Coyle, Scott Kelly, John McNair, and Butch Cobb. Gary Gunstrom edited the manuscript and Marla Trollan produced the final document.

PROJECT SPONSORSHIP

This report is in fulfillment of Delivery Order #41-0109-1-0207 to the U.S.D.A. Forest Service Procurement Office, P.O. Box 21628, 709 W. 9th Rm 267, Juneau AK 99802-1628.

PREFACE

This report constitutes the documentation requested by the U.S. Forest service, U.S.D.A., in fulfillment of the delivery order 41-019-1-0207. The contract language stipulated that the funds be used to conduct salmon enhancement studies on transboundary rivers (Stikine and Taku) under Authority PL 101-512 and the Sikes Act, PL. 93-452. The rational for the project stems from an agreement under the U.S./Canada Pacific Salmon Treaty (Treaty), which states that "the two parties shall cooperate in management, research, and enhancement." During February 1989 meetings in Portland, an "Understanding between the United States and the Canadian Sections of the Pacific Salmon Commission concerning Joint Enhancement of Transboundary River Salmon Stocks" was signed in which the parties agreed to specific joint enhancement projects on the rivers, and to share the costs. These funds were used to help implement the U.S. portion of the agreement for the federal fiscal years 1992 and 1993.

Under the agreement, a portion of these funds were used to help pay for costs associated with rearing sockeye eggs and fry at the Central Incubation Facility at Port Snettisham Hatchery, and to transport the sockeye fry back to their natal areas. As adults, the enhancement fish are expected to contribute to the commercial fisheries of both countries. To evaluate the success of these enhancement efforts and to meet harvest sharing agreements between the U.S. and Canada, funding was also included for the implementation of an otolith mass marking system. Under this system, all fish reared at the facility were subject to a sequence of temperature manipulations to place identifying bands on the sockeye's otolith (a small crystal that resides in the fish's brain capsule). Because thermal marking of otoliths is a new technology, these funds supported research and start-up costs for the establishment of an otolith laboratory, in anticipation of the first enhanced adults returning in 1993. This report summarizes the efforts to develop thermal mark recovery methods that were achieved during the period of this contact.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

SUMMARY

Since its inception, the Transboundary River Enhancement Project has contributed to the rearing of 26 different sockeye groups totalling over 35 million fry from the Stikine and Taku Rivers. The sockeye eggs were collected from the spawning grounds, transported to Snettisham Hatchery where they were incubated, and the fry then released back into areas of the rivers identified as being under-utilized in their rearing capacity. The first large numbers of returning adults from these efforts is expected in 1994. In order to monitor the success of the enhancement program, and to help allocate the catches of the returning adults to meet harvest sharing agreements, thermal mass marking of otoliths was chosen as the method to distinguish these sockeye from each other and from wild stocks. At the time of the initial releases of fry, the technology to efficiently recover thermal marks in the microstructure of fish otoliths had not been fully developed. One of the primary goals of this funding was to advance the methodology for extracting otolith information in a rapid and a cost effective manner such that it can be used to meet the Treaty objects. To achieve this goal, the project was broken down into a series of sub-objectives that provided a series of bench mark steps. The subcomponents of the project included finding ways of improving the quality of the thermal marks imposed on the otoliths in the hatchery, developing material processing methods and optical detection approaches for identifying thermal marks in otoliths, and developing the appropriate sampling and statistical methodology to provide information on the composition of the stocks in the commercial fisheries.

With the funding provided by this contract, progress has been made on setting a program to meet the Treaty objects when the adult sockeye return, though some work still remains. The activities performed under this contract included conducting an extensive review of otolith microstructure literature, with emphasis on thermal marking research. A summary of the information is provided in this report. In addition, steps were made to establish standards for future thermal marking efforts by undertaking a cooperative study with the University of Alaska Fairbanks to conduct thermal marking experiments at a local hatchery. The rationale for this project was an indication that the quality of thermal marks in early releases was not uniform. The project is currently on-going and is funded, in part, by the Alaska Science and Technology Foundation. A final report is due January 1994. Tentative results from the study indicate a need to avoid the hatching event during the period of thermal marking, and a cycle of 48 hours hot water/48 hour cold water will produce a strong thermal ring.

A number of approaches were also evaluated for processing otoliths to detect thermal marks. At this point, it appears the most effective approach to start with is the most simple: manual grinding and polishing individual otoliths to expose the microstructural core. Processing rates based on manual processing appear adequate for determining the composition of catches provided there is an adequate sampling program in place. Pattern recognition methods to detect thermal marks based on optical imaging technology remains a long term goal. However, on the immediate horizon the human eye appears to be the most efficient and accurate means of distinguishing thermal marks. Sampling protocols will be based on efforts necessary to achieve random samples, and statistical inference about the composition of catch through thermal mark recovery will be based on a binomial distribution. For every time-area strata of

interest, a target sample size of approximately 400 otoliths will be adequate to achieve a 95% confidence interval that is within $\pm$ 5% of the estimated enhanced contribution, even under the scenario of 50% enhanced fish: 50% wild stocks. In practice, samples needed to achieve that same precision will be less if the proportion of enhanced fish is less than 50%. A two stage sampling approach will likely be used. An initial subsample from the 400 processed otoliths will be used to provide a more immediate estimate of enhanced contribution to the fisheries managers such that they can use the estimates to make decisions on an in-season basis. These initial estimates will be used to determine the number of samples necessary for further processing on a postseason basis to achieve a set degree of precision around the estimate of enhanced contribution.

After two years of development, the program appears to have good prospects for working. Early recoveries of thermally marked sockeye smolts from all of the enhanced areas, indicate that the marks are retained and, in general, are recoverable. In addition, thermal marks were detected in the otoliths from precocious male sockeye returning early to one of the systems in 1992, indicating that the enhancement efforts will likely be a success at least in some systems. While some problems are anticipated with recovering the marks from some adult returns, on the whole, it is expected that the objectives of these enhancement efforts will be meet.

INTRODUCTION

The U.S. and Canada negotiated agreements between 1988 and 1990 on the joint enhancement of sockeye salmon stocks in the transboundary Stikine and Taku Rivers. Evaluating the success of the enhancement efforts and monitoring of the agreements concerning harvest sharing guidelines requires a reliable method of identifying the proportion of enhanced fish captured in the commercial fisheries. The Transboundary Technical Committee of the Pacific Salmon Commission, in 1989, recommended the use of thermally-induced otolith marking as a method to mass-mark all fish produced for the cooperative enhancement projects. This new method takes advantage of the unique characteristic of otoliths to record abrupt temperature fluctuations in their microstructures (microstructures referring to features which can only be observed with the aid of microscopes). Manipulating hatchery water temperatures during embryonic stages can result in a series of banding patterns laid down in the otolith microstructure. Given proper preparation, these patterns can be observed in the otoliths of the returning adult fish. The marking rate of fish exposed to the thermal manipulation approaches 100%. This characteristic, alone, suggests that it is a promising method to meet Pacific Salmon Treaty objectives. Coordinating the implementation of the marks and identifying the marked fish in a rapid and efficient manner from the commercial catch remain the primary challenges of the project.

This report contains a summary of the activities used to achieve the project objectives during the contract period. A literature search on published and unpublished information on the characteristics of otolith microstructures was conducted. A summary of that research is provided, along with a review of previous efforts and attempts to thermally mark salmon in Alaska. This background information provided the basis for formulating a series of objectives with which to achieve project goals. A discussion of these objectives is provided along with an outline of the project's components. Under the results and discussion section is a review of the activities and approaches taken for each component. These include ways to improve the quality of the thermal mark, options for mass processing otoliths to recover thermal marks, and sampling protocols that are being considered to implement the technology to meet the Treaty goals.

Background on Otolith Research

Otoliths are biogenic crystals found in the brain capsules of most species of fish. They are composed of calcium carbonate (of the aragonite crystalline form) growing within an organic matrix. The extracellular characteristic of their growth gives them the ability to serve as sensitive (though complex) barometers of ambient conditions. During normal growth, protein and crystal deposition is incremental, though off-phase, generally following a 24-hour cycle. The protein is optically dense in comparison with calcium. Changes in the ratio of calcium and protein give the appearance of concentric light and dark rings when viewed with transmitted light. In general, these are referred to as daily growth increments (Compana and Nelson 1985). The rate of otolith crystalline growth is, to a large extent, dependent on metabolic rates and is thus temperature dependent. During warmer periods and under normal conditions, individual aragonite crystals are long and narrow in form, giving the appearance of large spaces between rings; during colder temperatures the crystals are short and broad, resulting in narrow ring spacing (Gauldie and Nelson 1989). If temperatures undergo an abrupt drop, or if the fish is stressed in some way, precipitation of calcium carbonate out of solution may cease entirely. The actual mechanism may involve a pH shift within the endolymphatic fluid (Gauldie and Nelson 1989), though whatever the cause, the result appears as a deep discontinuity in the calcium carbonate crystals, leaving a protein rich zone. Under transmitted light microscopy these zones have the appearance of a prominent dark ring which is commonly referred to as a "check mark". By manipulating the temperature in a hatchery it is possible to induce the appearance of these check marks in a pre-planned pattern. The physiological mechanism which forms the check marks is sufficiently ubiquitous that all fish subject to the same temperature drop will form the mark. However, the appearance of a mark may vary depending on the size of the fish and whether intrinsic processes are also laying down check marks. Scheduling the temperature drops such that the marks appear in an orderly, "unnatural" fashion provides a means of separating hatchery stocks from wild stocks. The appearance of the marks is further enhanced by the relatively uniform growing conditions of a hatchery which reduces the contrast of the daily increments, and provides a clear background against which the marks appear.

The literature reports a number of studies in which reared fish have been successfully marked (e.g., Campana and Nelson 1985). Most of the studies, however, dealt with the research goal of trying to identifying the exogenous sources which control otolith microstructure growth. Mosegaard et. al. (1987), reported some early experiments in Sweden to encode patterns on otoliths using temperature and light changes as a mass marking tool. In the U.S., Brothers (1985) recommended to the Great Lakes Fishery Commission that the ability to manipulate otolith patterns be used to identify hatchery raised lake trout. Eric Volk, in conjunction with colleagues at the Washington Department of Fisheries (Volk et.al. 1990) later applied this concept to Pacific Salmon. After successfully inducing banding patterns in chinook, chum and coho salmon, they have managed to detect the mark in some initial returns of the released fish (Volk personal communication).

Alaskan Research

In Alaska, strong interest in the thermal mass marking approach has lead to a number of preliminary studies which have confirmed its potential as a mass marking tool. Efforts have also extended to high production releases. A brief chronology of these efforts is provided.

In 1988, a pilot project, in which several thousand pink salmon were thermally marked and coded wire tagged, was conducted by the Salmon Gulch Hatchery operated by the Valdez Fisheries Development Association (VFDA) (Table 1). The thermal manipulation was conducted by mixing water from two sources with different ambient temperatures. The adults returned in 1990. Sub-samples were taken and successfully identified as thermally marked or unmarked by two independent otolith laboratories.

Table 1. Summary of thermally marked salmon releases in Alaska.

Species	*Hatchery*	*Brood Years*	*Number of Groups*	*Releases (millions)*	*Recovery Plan*
Pink	VFDA	88	1	-	Pilot study
Pink	Auke Creek	89, 90	2	-	Pilot study
Pink	DIPAC	90, 91,92	3	131.0	Coop.
Chum	DIPAC	91, 92	4	127.0	?
Chum	NSRAA	91	1	10.0	?
Sockeye	**Snettisham**	**88 thru 92**	**26**	**35.0**	**US/Can**
Sockeye	Trail Lakes	90, 91, 92	11	22.0	?
Coho	DIPAC	91, 92	2	2.0	?
Chinook	DIPAC	91, 92	3	0.5	?
		TOTAL	52	327.5	

The Alaska Department of Fish and Game, at its Snettisham Central Incubation Facility (CIF), experimented with various marking cycles in sockeye salmon in 1988 and released the smolts into Speel Lake. No significant adult returns are expected from this first release. The studies have continued at the facility, and in 1989 the program was expanded to mark 5.4 million sockeye from three stocks using three different patterns. The sockeye were released to Speel, Crescent, and Tahltan Lakes, and the first contributions of these fish to the commercial fishery should occur in 1993 (Crandall et al. 1990). Juveniles from the first releases were examined to confirm that the mark did, indeed, appear on the otolith. During the ensuing years, additional sockeye stocks have been marked at the CIF with the presumption that the adults will prove separable upon return. A schedule of planned marks and dates of return are provided in Appendix 1.

The application of thermal marking at the CIF has thus proceeded from an experimental to a production scale (Table 1), despite the fact that no adult sockeye otoliths have been examined to evaluate the readability of the marks or to determine possible conflicts with natural marks. An examination of some of the marked otolith samples collected at the hatchery prior to the releases suggests that the marks from some of the stocks maybe difficult to recover in the adults when they return in 1994. Developing the methodology to identify these marked individuals amongst the other returning sockeye adults will be a challenge to our otolith laboratory in Juneau.

Other thermal marking experiments in Alaska included a pilot study conducted with pink salmon at the Auke Creek Hatchery in the fall of 1989 (Munk and Smoker 1990) (Table 1). The cooperative project between the National Marine Fisheries Service, the University of Alaska, and the Department of Fish and Game saw approximately 100 returning adults in September 1991. These fish were also marked with fin-clips and coded wire tags to facilitate identifying them upon their return. In 1990, Douglas Island Pink and Chum Inc. (DIPAC) thermally marked all of its pink salmon for release (30 million) (Table 1), with a subsample that was coded wire tagged (Munk and Smoker 1992). The adults returned in 1992. A sampling program was conducted to collect the otoliths in mixed stocked areas. A portion of them were examined in the winter of 1992 - 1993 to develop production rates for processing otoliths.

In 1991 and 1992, DIPAC continued to mark its pink salmon and, in addition, marked all of its chum, coho and king salmon (Table 1). A cooperative agreement was reached with ADF&G in 1993 to analyze the otoliths from pink salmon returning to the mixed stock areas in 1993. The purpose of this study is to develop the capability to provide inseason estimates of the proportion of hatchery to wild fish in the return. A report evaluating the success of the project is due in January 1994.

The Northern Southeast Aquaculture Association's (NSRAA) Hidden Falls Hatchery marked 10 million chum salmon in what was initially a planned release in its terminal harvest area (Table 1). No mixed stock concerns were identified at that time, and the marking was undertaken by the hatchery primarily to determine its ability to control its temperature manipulations. Our lab collected voucher specimens from the hatchery but made no commitment to process the otoliths upon return. Since that time, a permit to transfer the fish to Boat Harbor near Juneau, was granted, raising the possibility of conflicting marks with the DIPAC chum. Subsequent review of the voucher collection suggested the marks were separable.

Trail Lake Hatchery, in the Cook Inlet Region of Alaska, has also conducted thermal marking projects on sockeye salmon (Table 1). The hatchery uses two water sources with a temperature differential to conduct its marking. There currently is no plan to identify these marked fish in the commercial catches. The primary purpose in marking is to determine the numbers of hatchery smolts surviving after their release.

OBJECTIVES

The project objective is to develop the methodology to determine the contribution of thermally marked hatchery sockeye to the commercial fisheries. The purposes of acquiring this information are to 1) aid fishery management decisions on allocating fishing effort between wild and enhanced transboundary sockeye, and 2) determine the success of these enhancement projects in meeting U.S./Canada Treaty goals.

To achieve the objective, a laboratory will be established that will focus on two activities: 1) documenting pattern variation in otoliths of thermally marked sockeye prior to their release into the wild, and 2) develop and implement the methodology by which the marked fish can be recovered accurately and quickly from the commercial fisheries. In addition, the lab also needs to address long range goals for lab expansion by identifying new equipment purchases, and the training of personnel in otolith processing.

A series of sub-objectives were identified as means to achieve the overall project goal and provide a series of benchmarks with which to evaluate progress. The following outline presents the break down of the project objectives.

Objective Outline

I. Collect and process voucher specimen otoliths from thermally marked juvenile sockeye reared at the Snettisham CIF.

  A. Document and quantify pattern variation of thermal marks within and between different brood years and stocks, in preparation for the returning adults.

  B. Process otoliths of sockeye smolts from rearing lakes to evaluate the success of transplanted enhanced sockeye (may require specific funding).

II. Develop the capability to mass process adult otoliths

  A. Use available thermally marked adult pink salmon otoliths to determine production goals and detection rates.

1. Process otoliths from "known" thermally marked pink salmon for the purpose of determining a "readability" rate.

2. Process otoliths from marked pink salmon returning to the Hawk Inlet mixed stock area for the purpose of determining optimum handling procedures for mass processing otoliths under a "real time" basis.

B. Evaluate other options for extracting otoliths and exposing the microstructures.

III. Determine target sample sizes for otolith collection and otolith processing.

A. Develop a data management system to coordinate sample collection, storage and the dissemination of results.

IV. Apply pattern recognition approaches to aid in distinguishing marks and improving mark quality.

A. Develop image processing routines for automated thermal ring detection and measurements.

B. Develop a model of otolith growth to aid in determining appropriate marking schedules and identify limits to the number of marks available.

1. Collect CTU data and otolith measurements from marked fish.

2. Conduct joint research with hatcheries to experiment with different temperature schedules.

V. Provide recommendations for developing a long-term production goals and marking procedures.

DISCUSSION

Collecting Voucher Specimens

Documenting the thermally induced marks of released fish is an ongoing responsibility of the lab which requires close cooperation with hatchery personnel to obtain representative samples of the mark prior to release of fish. Such specimens serve as voucher collections, and are the only way the lab will be able to identify returning marked adult salmon. Appendix 1 contains a list of the 26 different releases of sockeye which have occurred to date. A sample of voucher specimens were collected from each incubator that was used for each of the reared groups.

A subsample of otoliths from the voucher specimens are processed prior to releasing the fish back into the lakes. The purpose is to determining if there are problems with any of the marks and to quantify the mark variation within and between different groupings. Research will be conducted to develop methods of quantifying pattern variation in the otoliths in a consistent manner by using image processing. Initial variables to be examined include ring count and ring spacing, with a ring defined as a specific change in luminescence above a minimum threshold value across a specific distance on the otolith (i.e., a dark ring against a lighter background as viewed with transmitted light microscopy). Temperature records taken during the development of the fish will also be examined to help identify unexplained, or unplanned rings that may appear in the voucher specimens.

A subsample of voucher otoliths will also be processed immediately prior to the return of the adults. This will give the individuals doing the processing the ability to develop a "search image" on the patterns they are likely to encounter in the adult otoliths from the mixed stock fishery. In addition to the voucher collections, samples from wild stocks will be examined to determine the "background" of patterns against which the marks will be detected.

Developing Mass Processing Methods

Options for removing otoliths from salmon on a large scale include removing otoliths in the field as part of sampling, or collecting the heads and removing the otoliths in the lab, or a combination of the two. It will be difficult to streamline field collection beyond the "knife and tweezers" approach. Training, however, can make dramatic improvements on the speed with which otoliths can be removed. We anticipate working with field personnel to develop standardized approaches. Initial estimates are a dissection rate of approximately 75 otolith pairs per hour per individual.

The otoliths need to be cleaned prior to mounting on glass slides for further preparation. Soaking in alcohol seems to be adequate in most instances, though bleach soaking and ultrasonic cleaning are options to be explored. After cleaning, the otoliths can be embedded in resin blocks to facilitate controlled grinding, or adhered to glass slides with a thermal plastic resin for more rapid manual processing. Initial estimates to mount individual otoliths on glass slides are approximately 25 per hour.

Options for exposing the microstructural core of the otolith is one of the most technologically challenging aspects of the project. Other research applications involving otolith microstructures do not require large numbers to be processed. There appears to be no model to turn to in developing mass processing methods, thus considerable time was devoted to experimenting with ways to expand the lab's production capabilities.

Though the lab has been actively looking for alternatives, we are currently operating under the assumption that physical removal of the overlaying otolith material, through grinding and polishing, is the only way to expose the microstructures of the otoliths and allow viewing with transmitted light. This is essentially an adaption from petrographic methods used to thin-section minerals and other hard substances. A couple of unique features of otoliths present difficulties not generally encountered in petrographic work: 1) The location of the thin section must be precise, as the information contained in the microstructure occupies a thickness of approximately 20 to 50 microns (though that is still to be determined) and, 2) otoliths are not identical in size or shape even between sagittal pairs taken from the same individual. For instance, the variance component of otolith shape within an individual is almost half of variance between individuals. Trying to expose the microstructures en mass, given this variability, will result in a number of otoliths which are either over- or under-ground. It will remain a challenge to try to keep these numbers to a minimum without investing additional time in processing.

For general applications in the lab, using the sagittae otoliths, half-sectioning will generally be adequate. With half-sectioning, only one surface of the otolith is ground to expose the microstructure, as opposed to thin sectioning in which both sides of an otolith are removed. Half-sectioning is preferred because it is less labor intensive and, provided the otolith is thin enough, adequate amounts of light will still pass through for detecting patterns in the microstructure using conventional transmitted light microscopy. For large otoliths such as found in adult chinook, the thickness of the otolith may necessitate a thin-section approach when viewing.

Half-sectioning is best accomplished by grinding down the proximal face of the otolith (which contains the sulcus groove). More calcium carbonate is deposited on the proximal surface, than on the distal surface during the process of otolith growth when the adult sockeye are out at sea. In general, leaving the distal face intact and removing the proximal face to expose the microstructural core will leave the otolith thin enough to view with transmitted light. Morphometric research will be needed to quantify the maximum thickness that can be left, and to identify, on average, the amount of material to be removed. Examination of a small sample of pink salmon otoliths indicates that 250 to 400 microns should be removed to allow the exposure of the central core. Under some scenarios, the amount of material removed can be controlled through micrometer adjustments on the grinding equipment, or an individual processing the otoliths will get a "feel" for his or her removal rates and learn when to stop grinding. Appendix 3 contains a digital image of a thermal marked adult pink salmon otolith that was examined in preparation for the return of adult sockeye. Appendix 4 contains the digital image of a thermally marked sockeye fry from a group that was released as presmolts into Crescent Lake in 1991.

Grinding otoliths can be accomplished manually or via automated or semiautomated means. Our laboratory will initially be using manual methods to expose the microstructural core. Each otolith will be mounted on separate petrographic slides with thermal resin and, using grinding wheels and increasingly finer grits of silicon carbide and alumina oxide polishing powder, trained personnel will remove the overlaying otolith material and polish the scratches off the remaining surface to allow sufficient viewing with transmitted light. This will be a labor intensive process which will require practice and training to develop efficient processing skills. Current estimates indicate that a trained individual can manually process an otolith within four minutes and make a determination on the presence or absence of a mark.

Automatic grinding approaches will be considered for future lab expansion. Automatic grinding machines incorporate the ability to produce consistently thin sections of a precise thickness. The otoliths are ground and polished on one side en mass, then manually flipped over and ground and polished on the opposite side. The quality of preparation is high; however, a considerable amount of time is involved in setting the equipment. Volk et al. (1990) describe the this approach.

ADF&G's limnology lab in Soldotna is exploring the use of a semiautomated approach to mass processing and has projected approximately 200 otoliths could be processed per day. With the semiautomated method, half-sectioning is used, and 100 otoliths at a time can be processed. During the grinding process the otoliths are viewed repeatedly to see if the microstructures of the otoliths are exposed and whether determinations can be made on whether they are marked or unmarked. The process continues until all the otoliths mounted have been ground through. The otoliths are essentially destroyed in the process; other than photo documentation, or a careful examination of the remaining sagittal pair, there is no record of the microstructural pattern to reference.

Alternative approaches to grinding otoliths and using transmitted light microscopy to detect thermal marks have been considered. Some methods explored include otolith decalcification and microtome sectioning, high pressure water-jet sectioning of otolith wafers, and confocal microscopy of whole otoliths. Future developments or technological breakthroughs may provide opportunities to revisit these methods.
Precluding any technological breakthroughs, improvements in the grind and polish approach will likely be made on an incremental basis, and as a result of trial and error. Under this scenario, the best approach will be to initially maintain a degree of flexibility. Various pieces of equipment will be needed with which alternative preparations may be tried and evaluated. Treating these alternative approaches as semi-formal experiments will provide a degree of methodology that may lead to improvements. As in any experiment, data will be needed to provide the raw material for evaluation. Initially, we will gather basic morphometric data on salmon otoliths, e.g., means and variances of whole otolith shapes (broken down to sockeye of various age classes.), as well as the shapes of the central primordia. Otoliths that have been successfully thin-sectioned to various degrees of clarity of their central cores, will also be studied to determine their thickness and surface smoothness. Quantifying our specimens morphologically from their raw shape to the final product within set tolerances will give us the tools needed to implement a quality control program. Including data on the speed of processing, labor, and associated costs, will give us a means to determine the advantages or disadvantages of alternative approaches.

To help develop the methodology to rapidly process and recover thermal marks, collections of pink salmon from a mixed stock area will be taken through a cooperative agreement with Douglas Island Pink and Chum Inc. in Juneau, Alaska. After the fish are collected, the otoliths are removed, mounted on glass slides, and manually processed to determine the proportion of the thermally marked fish in the catches. This project, taking place in the summer of 1993, will be invaluable help in developing methodology for preparing for the returns of adult sockeye in 1994. Blind tests using known marked and unmarked otoliths will be conducted while processing these samples to evaluate the success in recovering thermal marks.

Sample Size Determination

Sample sizes for otolith processing will be considered on a project by project basis, but in most management mixed stock scenarios a target of 400 otoliths will be collected for every time/area strata of concern. Four hundred is the size necessary to achieve a 95% confidence interval that is within 5% of the estimated proportion under a scenario of 50% enhanced fish. This is based on the binomial distribution and provides an upper figure to the number of otoliths that would need to be processed. If the actual percentage of enhanced fish in the catch is smaller than 50%, then fewer samples are need to achieve the same degree of precision (Figure 1).

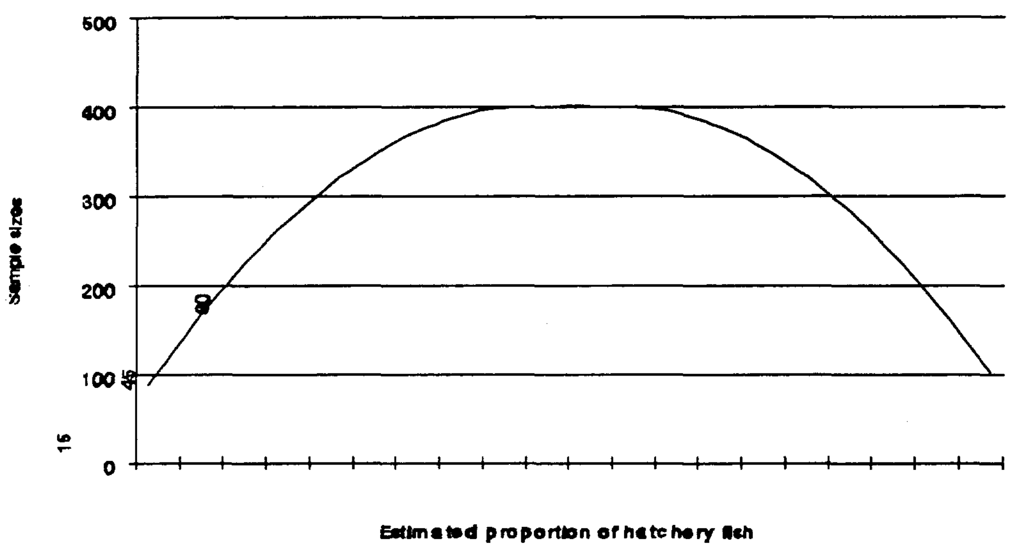


Figure 1. Sample sizes necessary to achieve upper and lower 95% confidence intervals that are within $\pm$ 5% of the estimated proportion of hatchery fish. Calculations are based on the exact binomial method.

Sample sizes necessary to achieve a set degree of precision are relatively independent of the size of the population that was sampled. This is illustrated in Figure 2, where the 95% confidence intervals for three sample sizes are almost constant for a large range of population sizes. Estimating the composition of a large population can be done with relatively small samples. The requirement, from a sampling perspective, is that the specimens are collected randomly, and that every fish in the population of interest has an equal probability of being a hatchery fish or a wild fish. While small samples are desirable from a processing perspective, this creates a burden to assure that the specimens are representative of the population.

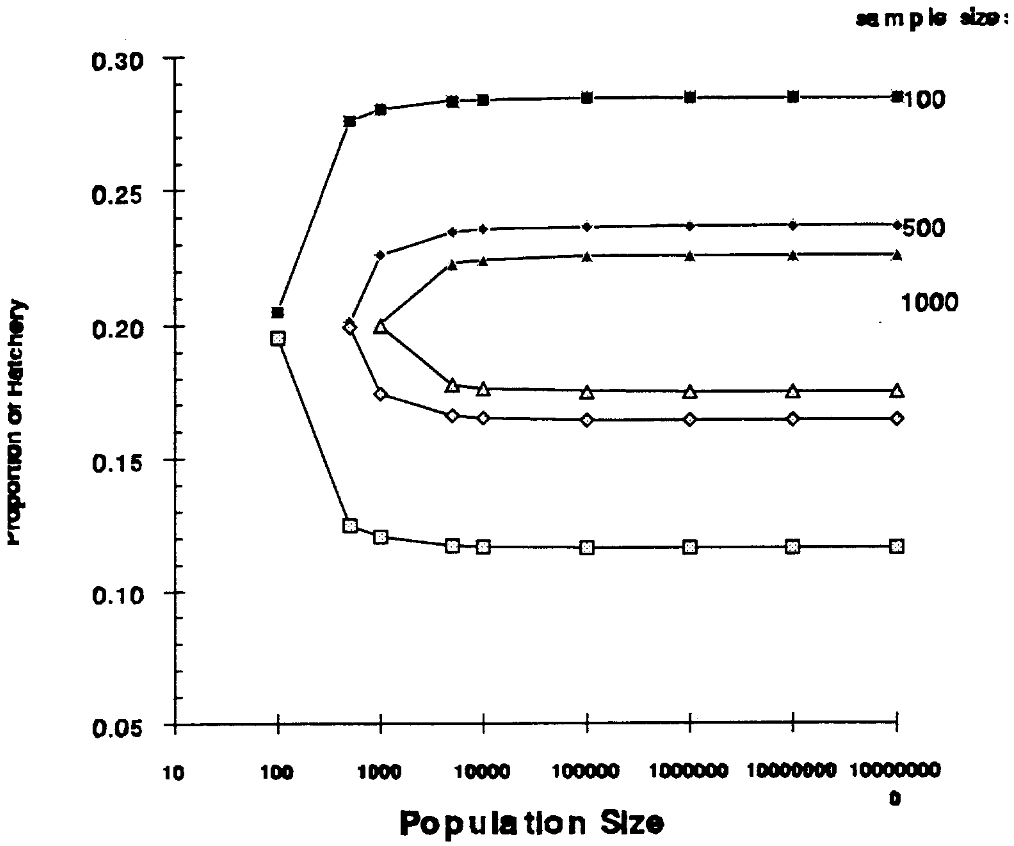


Figure 2. Upper 95% confidence range (black symbols) and lower 95% confidence range (open symbols) for sample sizes of 100, 500, and 1,000, around an estimate of 20% hatchery contribution over a range of population sizes.

A sequential sampling approach will be used to determine the numbers to process in the lab. In most cases, an initial 100 otoliths from the random sample of 400 will be processed and examined. Based on an *a priori* determination on an acceptable level of uncertainty in conjunction with the initial estimate, the number of additional otoliths that need processing can be calculated. Included in this decision process will be the concerns over the abundance of wild stocks and the relative urgency of needing a more precise estimate. Other factors include competing commitments of the lab's personnel and time, funding considerations, and the overall objective of the sampling. In general, we envision a two stage approach where a rapid turn around of information is needed for inseason management decisions, and then a less urgent need for postseason analysis of the proportion caught. Appendix 2 contains a flow chart of this approach. The Department of Fish and Game's Integrated Fisheries Data Base (IFDB) is the data base the otolith lab will eventually use to provide information to the managers.

Sampling needs for other laboratory projects, including voucher collection and research sampling, will generally be determined on the basis of estimating the amount of actual or estimated variance within a sampled population. This amount will then be used to determine, on an objective basis, our ability to distinguish significant differences in the sampled populations with a set degree of precision. Sampling considerations will be evaluated on a continuing basis, and alternative approaches such as Baysian analysis or hypothesis-driven sequential sampling will be explored.

Pattern Recognition and Model Development

Upon the return of thermally marked adult salmon, the pattern that was imposed on the microstructure of the otolith needs to be detected against a background of natural patterns. The human eye is currently the most sophisticated and cost-effective way to identifying complex patterns. However, developing pattern recognition algorithms to identify thermal marks could provide a means to eventually automate the mark detection process and provide a degree of objectivity to the recovery program. While developing a "bar code" type reader to detect thermal marks may be a long term goal; in the interim, a series of intermediate steps using image enhancement and pattern recognition algorithms can be used to help the laboratory technician identify thermally marked otoliths.

Developing algorithms to identify thermal marks can also help in finding ways to improve the quality of thermal marks. Analysis of the voucher collections will be used to provide recommendations on ways to improve the quality and consistency of marks. A mathematical model of otolith growth based on the temperature data and ring size and ring spacing can be constructed. From this model, templates of temperature cycles can be designed for future programs that optimize the cost of heating the water while providing marks that are clearly discernible from other marks and background patterns.

The concept of "marking windows" will also be addressed through a modeling approach. On an individual basis, a marking window is that period of time between the fusion of otolith primordia and hatching. The lab is concerned that the hatching event will produce a mark in the otolith that is indistinguishable from a planned thermally induced mark. We need to determine the variation in patterns that result from inducing temperature fluctuations during the hatching period and what cost this may have on our ability to distinguish these cases from other planned or natural patterns. If a hatching event is to be avoided in future markings, we need to determine a population size window when fish are present at different developmental stages. Since the cumulative temperature required to trigger hatching is not constant with respect to temperature, and temperature changes are the coinage used to create marks, developing a model that predicts when hatching occurs would be a useful tool in determining marking windows for a population. The lab will investigate the potential of using marking windows that occur after the hatching period. The lab secured additional funding in 1992 to engage in a cooperative study with the University of Alaska to conduct experiments for inducing thermal marks. These results will be used to address the above needs. The scheduled completion date is the end of 1993. Tentative results from the study indicate a need to avoid the hatching event during the period of thermal marking, and a cycle of 48 hours hot water/48 hour cold water will produce a strong thermal ring.

Long-term Developments

Both research and training will be required to continue the development of thermal marking,. Research will be required on production level sampling, otolith processing, and mark detection methods. Samples are being collected of wild adult sockeye that are returning to the fisheries, along with the first of the early releases of thermally marked sockeye. In addition, thermally marked pink salmon adults are also available from the commercial fisheries. These specimens are serving as fodder for practicing and training ADF&G port samplers on techniques for removing otoliths from the commercial catch and in the field during test fisheries. The otoliths removed can then be used for research and for practicing processing methods.

In 1994, 11 groups of thermally marked sockeye adults are expected to return (Appendix 1). Three of the groups will be five-year-old sockeye which will comprise 70% to 90% of the return. The return year for the five-year-olds are indicated by the shaded boxes in Appendix 1. In 1996, these numbers will increase to 21 separate groups, with 6 groups being composed of the dominant age class. This contract has provided the basis for developing methods in preparation for these returns. The next step will be to work closely with fisheries managers in developing strategies to make use of the information on enhancement contributions to aid inseason management. In addition, to prepare for a large number of samples, an inventory control system will be established to streamline the process of handling otoliths in the laboratory.

LITERATURE CITED

Brothers, E. 1985. Otolith marking techniques for the early life history stages of lake trout. Great Lakes Fishery Commission Completion Report, Ann Arbor Michigan.

Compana, S.E. and J.D. Neilson. 1985. Microstructure of fish otoliths. Can. J. Fish. Aquat. Sci. 42:1014-1032.

Crandall, K. C. Coyle, A. McGregor, and John Wilcock. 1990. Report from the work group on thermal induction of banding patterns in salmonid otoliths. *In* Geiger, H. and R. Wilbur (editors). Proceedings of the 1990 Alaska Stock Separation Workshop. Alaska Department of Fish and Game, Division of Commercial Fisheries, Special Fisheries Report 2, Juneau.

Gauldie, R.W. and D.G.A. Nelson. 1988. Aragonite twinning and neuroprotein secretion are the cause of daily growth rings in fish otoliths. Comp. Biochem. Physiol. 90A(3):501-509.

Mosegaard, H., N.G. Steffner, and B. Ragnarsson. 1987. Manipulation of otolith microstructures as a means of mass-marking salmonid yolk sac fry. Proc. V. Cong. Europ. Ichthyol. Stockholm, 1985: 213-220.

Munk, K.M., and W.W. Smoker. 1990. Temperature-induced marks in otoliths of pink salmon embryos. University of Alaska, School of Fisheries and Ocean Sciences, Juneau Center for Fisheries and Ocean Sciences, JCFOS #90-01. Juneau.

Munk, K.M., and W.W. Smoker. 1991. Temperature-induced mass marking of pink salmon otoliths. Production trail at Gastineau Hatchery. Final report to DIPAC, Inc., and Marine Advisory Program. University of Alaska, School of Fisheries and Ocean Sciences, Juneau Center for Fisheries and Ocean Sciences, SFOS-JCFOS #91-02. Juneau.

Volk, E.C., S.L. Schroder, and K.L. Fresh. 1990. Inducement of unique otolith banding patterns as a practical means to mass-mark juvenile Pacific salmon. American Fisheries Society Symposium 7: 203-215.

APPENDIX

APPENDIX 1

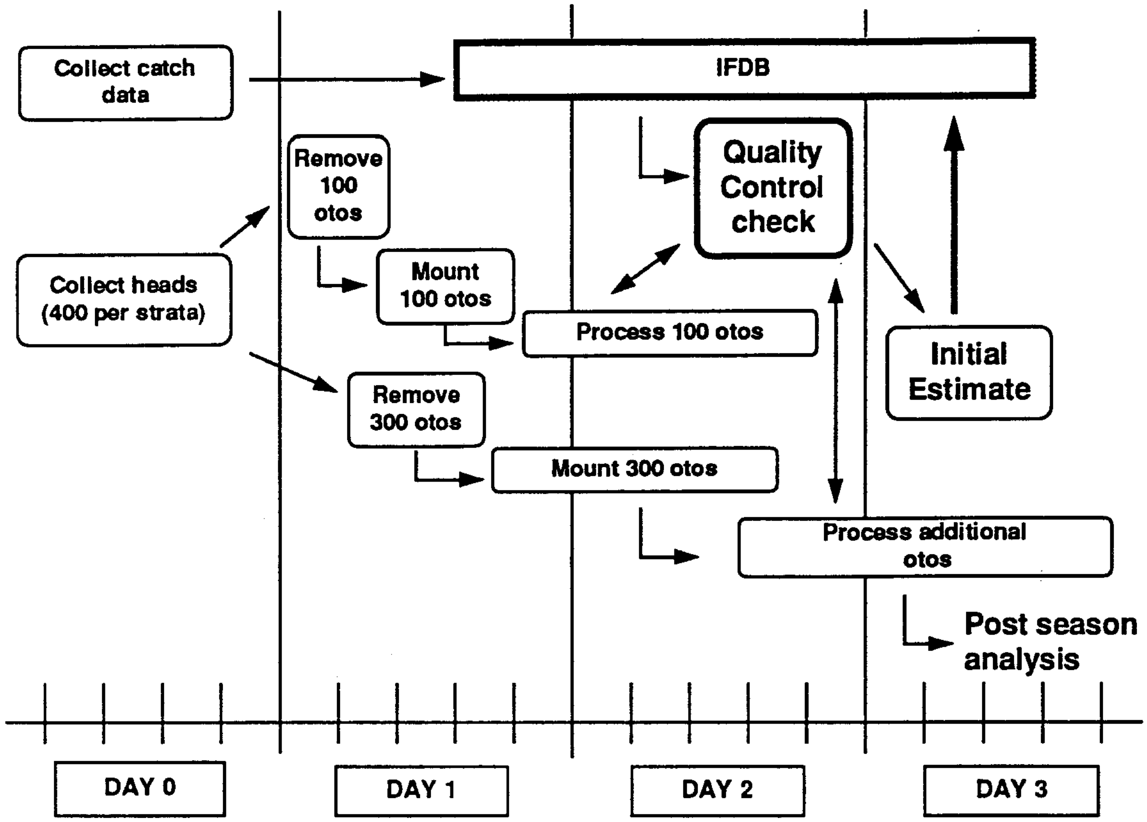
THERMAL MARKING PATTERNS OF U.S. AND CANADIAN SOCKEYE RELEASES

RELEASE GROUP			BROOD YEAR					RETURN YEAR: Ages 4-6						Return
Stock	Location	Stage	88	89	90	91	92	93	94	95	96	97	98	District
Speel	Speel	fry	3					3	3					11,12
Speel	Speel	fry	5					5	5					11,12
TAHLTAN	Tahltan	fry		4				4	4	4				6, 8
Crescent	Crescent	fry		6				6	6	6				11,12
Speel	Sweetheart	fry		8				8	8	8				11,12
TAHLTAN	Tahltan	fry			3				3	3	3			6,8
TATSAMENIE	Tatsamenie	fry			3				3	3	3			11,12
TRAPPER	Trapper	fry			5				5	5	5			11,12
Speel	Sweetheart	fry			7				7	7	7			11,12
Speel	Sweetheart	fry			4, 2				4, 2	4, 2	4, 2			11,12
Crescent	Crescent	fry,presmolt			10				10	10	10			11,12
TAHLTAN	Tahltan	fry				4				4	4	4		6,8
TATSAMENIE	Tatsamenie	fry				4				4	4	4		11,12
TRAPPER	Trapper	fry				6				6	6	6		11,12
TUYA	Tahltan	fry				6				6	6	6		6,8
Crescent	Crescent	fry				4, 5				4, 5	4, 5	4, 5		11,12
Crescent	Crescent	presmolt				4, 3				4, 3	4, 3	4, 3		11,12
Crescent	Crescent	smolt				4, 4, 3				4, 4, 3	4, 4, 3	4, 4, 3		11,12
TAHLTAN	Tahltan	fry					7				7	7	7	6,8
TATSAMENIE	Tatsamenie	fry					4 + 3				4 + 3	4 + 3	4 + 3	11,12
TRAPPER	Trapper	fry					7 + 3				7 + 3	7 + 3	7 + 3	11,12
TUYA	Tahltan	fry					5				5	5	5	6,8
Speel	Port Snet.	fry					5, 3				5, 3	5, 3	5, 3	11,12
Speel	Port Snet.	fry					3, 3				3, 3	3, 3	3, 3	11,12
Crescent	Sweetheart	fry					3, 5				3, 5	3, 5	3, 5	11,12
Crescent	Gilbert Bay	smolt					5, 5				5, 5	5, 5	5, 5	11,12

Example: 3,3 = 2 bands of 3 rings each, band interval = 2-3 times ring interval
3+3 = 2 bands of 3 rings each, band interval > 6 times ring interval

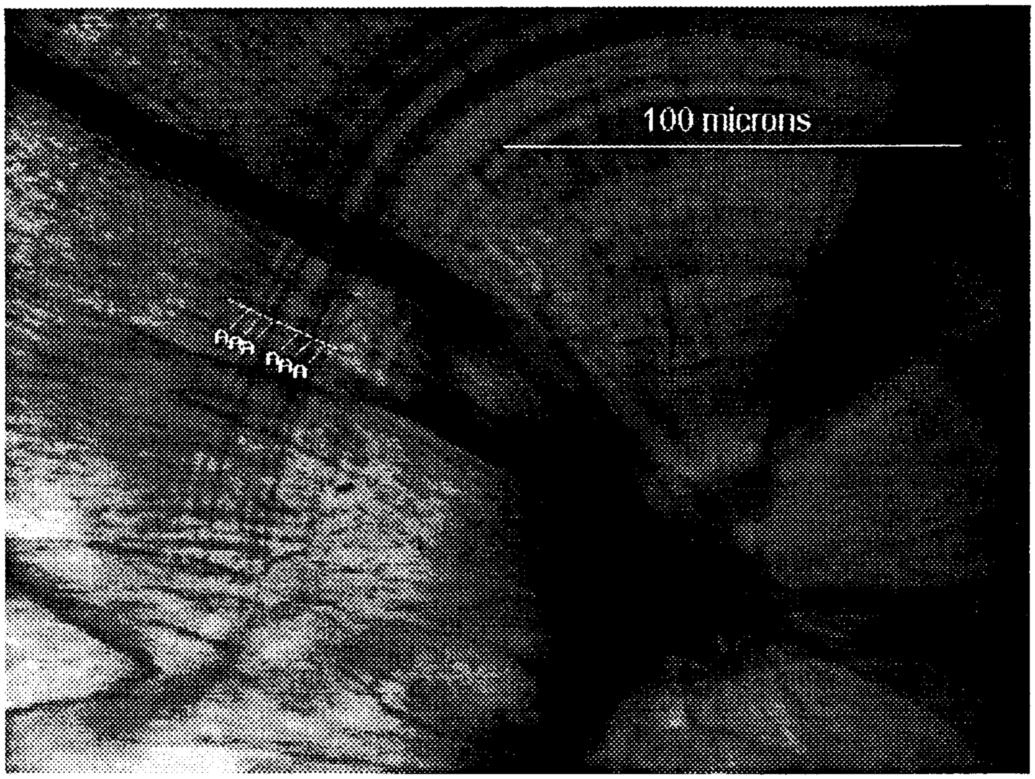
APPENDIX 2

In-season Processing of Otoliths



APPENDIX 3

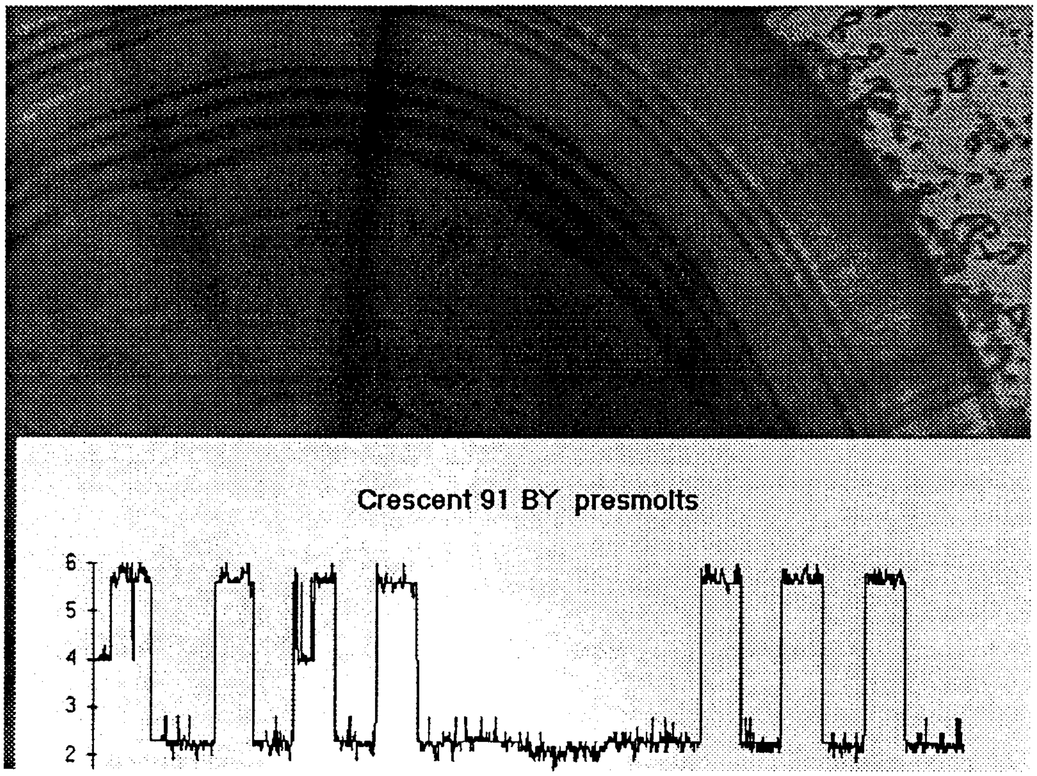
Photomicrograph of thermally marked adult pink salmon otolith.



Thermally marked adult pink salmon otolith with the "DIPAC 90 " mark (Munk and Smoker 1991) recovered from a mixed stock fishery in 1992 and processed in the Alaska Department of Fish and Game's Otolith Lab. Thermal rings are indicated with the letter A. The digital image was taken with a compound microscope using transmitted light at 200x. A 100 micron scale is included. The appearance of the microstructure is dependant upon the orientation of the crystalline bundles of the otolith which can scatter light, the location of dense proteinaceous regions which can absorb light, and by fractures running through the otolith which will deflect light and appear as dark swaths in the image. During removal of the overlaying otolith material by grinding, the optical characteristics change, resulting in a complex array of light and dark fields. The best location to view the thermal marks are in fields uninterrupted by fractures in which the crystalline bundles run perpendicular to the viewing plane. In those cases the "shadows" of the optically dense protein deposits associated with thermal marks appear in contrast to the uniform background of normal growth.

APPENDIX 4

Photomicrograph of thermally marked sockeye fry otolith with graph of temperature changes used to induce the thermal mark.



Otolith of a thermally marked sockeye fry from Crescent Lake 1990 brood year. The graph contains half-hour temperature readings over a four week period of thermal marking. Each temperature drop corresponds to a dark ring in the otolith microstructure.